Departement für Pferde, Klinik für Pferdechirurgie

der Vetsuisse-Fakultät Universität Zürich

Direktor der Klinik: Prof. Dr. med. vet. Anton Fürst, Dipl. ECVS

Arbeit erstellt unter der wissenschaftlichen Betreuung von

Dr. med. vet. Andrea Bischofberger, Dipl. ACVS & ECVS

Histological and biochemical characteristics of the articular cartilage of normal and osteoarthritic equine distal interphalangeal joints

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vorgelegt von

Andrea Bachmann

Tierärztin

aus Luzern, Menzingen (ZG) und Aristau (AG)

genehmigt auf Antrag von

Prof. Dr. med. vet. Anton Fürst, Dipl. ECVS, Referent

Prof. em. Dr. med. vet. Hans Geyer, Korreferent

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1 Summary

The aim of this study was to evaluate the glycosaminoglycan (GAG), water and collagen type II content of healthy and osteoarthritic equine distal interphalangeal joint (DIPJ) cartilage. Osteochondral samples were collected from 12 equine forelimbs (15.2±9.2 years). GAG and water content was analyzed at various regions of interest within the joint (dorsal/middle/palmar, condyle/intercondylar groove). Samples were histologically scored into three grades of osteoarthritis (OA) (normal, mild-moderate OA, severe OA). Denaturated collagen type II was analyzed immunohistochemically and the quality was scored using polarized microscopy following picrosirius red staining.

GAG content in the middle zones was significantly lower compared to the palmar zones. Water content in the middle zones was significantly higher compared to the palmar zones. There was no significant difference between the GAG and water content of the dorsal and palmar zones. GAG content decreased and water content increased with severity of OA. Immunohistochemically denaturated collagen type II fibers showed an increase in brown stain and loss of birefringence in polarized microscopy with increasing OA.

The topographical variations in the ECM of the DIPJ are probably due to the differences in load that act on the articular cartilage in this joint. GAG content decreased, and water content increased with higher grade of OA. All histological scoring systems were reliable and the score increased with higher grades of OA.

1. Zusammenfassung

Das Studienziel war es, den Glykosaminoglykan- (GAG), Wasser- und Kollagentyp II-Gehalt von gesunden Hufgelenken und solchen mit Osteoarthritis (OA) zu analysieren.

Knorpelproben wurden den Vordergliedmassen von 12 Pferden entnommen (mittleres Alter 15.2±9.2). Der GAG- und Wassergehalt wurde an verschiedenen Orten im Gelenk (dorsal/zentral/palmar; Kondylus/Kondylargrube) bestimmt. Alle Proben wurden histologisch in 3 Grade unterteilt (gesund, milde-moderate OA, schwere OA). Der Kollagentyp II-Gehalt wurde durch Immunhistochemie bestimmt. Die Qualität der Kollagenfasern wurde an Picrosirius Rot gefärbten Schnitten im Polarisationsmikroskop untersucht.

Der GAG-Gehalt war zentral signifikant tiefer verglichen zu den palmaren Regionen. Der Wassergehalt war zentral signifikant höher verglichen mit den palmaren Arealen. Zwischen dem GAG- und Wassergehalt der dorsalen und palmaren Regionen wurde kein signifikanter Unterschied festgestellt. Der GAG-Gehalt sank und der Wassergehalt stieg mit zunehmendem Grad an OA. In der Immunhistochemie stieg der Grad an Braunfärbung und die Kollagenfasern zeigten mit zunehmender OA keine Doppelbrechung mehr im Polarisationsmikroskop.

Die topographischen Unterschiede der GAG- und Wassergehalte im Hufgelenk sind möglicherweise bedingt durch Kräfteunterschiede, welche auf den Gelenkknorpel wirken. Mit steigender OA-Grade sinken die GAG-Gehalte und die Wassergehalte steigen. Die histologischen Scoringsysteme waren verlässlich und der Score stieg bei höherem OA-Grad.

2. Introduction

2.1 The distal interphalangeal joint

The distal interphalangeal joint, also referred to as the coffin joint, is a complex joint. It is composed of the middle phalanx (P2), the distal phalanx (P3) and the navicular bone (NB). Various ligaments (collateral ligaments, the deep digital flexor tendon (DDFT), distal digital annular ligament, collateral sesamoidean ligaments and the distal sesamoid impar ligament) provide stability to the joint (Figure 1). The specific configuration of this joint enables movement in the sagittal, frontal and transverse planes, thus allowing flexion and extension, lateromedial movements, rotation and sliding (Dyson, 2010). The distal interphalangeal joint, is a frequent source of lameness (Dyson, 1991; Kristiansen and Kold, 2007; McKnight and Posh, 2012).

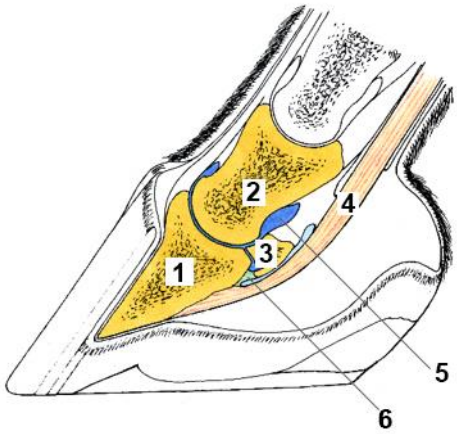


Figure 1*:* Anatomy of the distal interphalangeal joint

1) *P2= Middle Phalanx*
2) *P3= Distal Phalanx*
3) *NB= Navicular bone*
4) *DDFT= Deep digital flexor tendon*
5) *DIPJ= Distal interphalangeal joint*
6) *Bursa podotrochlearis*

2.2 Articular cartilage

2.2.1 Articular cartilage in general

Articular cartilage covers the ends of the articulating bones in synovial joints and has a central role within the joint. It enables a nearly frictionless movement, provides tensile strength and resistance to compressive forces. The condition of articular cartilage itself mainly determines joint health (Frisbie 2012).

2.2.2 Articular cartilage - gross appearance

Grossly articular cartilage appears smooth, milky or glasslike. In thinner areas the cartilage has a slightly pink appearance, because of the translucent subchondral bone plate. Although macroscopically the surface of the articular cartilage has a flawless impression, in electron microscopy small depressions with a diameter of 20-40 µm can be seen (Frisbie 2012; McIlwraith 2011). The articular cartilage is 1-4 mm thick. Thickness however depends on the joint itself and also differs within a joint. Because of the missing vascular, lymphatic and neural supply, the nutrition of the articular cartilage occurs solely via diffusion of the synovia (Frisbie 2012).

2.2.3 Articular cartilage - histological appearance

Histologically, the articular cartilage can be divided into four morphologically different zones: the hyaline cartilage, which can be divided in a superficial, intermediate and a deep zone and the calcified cartilage, adjacent to the subchondral bone plate. The tidemark is the border between the deep zone of the hyaline cartilage and the calcified cartilage. This region can be seen as a small line, under the light microscope (Figure 2) (Frisbie 2012). Although each zone has its specific morphological and mechanical properties, the boundaries cannot exactly be defined and appear rather blurred (Buckwalter et al., 2005). The superficial zone is the thinnest region (Martel-Pelletier et al., 2008), with the highest density of chondrocytes. The cells appear flattened and their long axis is aligned parallel to the surface of the cartilage. Collagen fibers located in this region are located parallel to the surface and the fibers have the smallest diameter. Chondrocytes in the intermediate zone in contrast appear larger and rather ovoid. The largest chondrocytes are located in the deep zone. In this area, the long axis of the cells is orientated perpendicular to the surface (Figure 3) (Frisbie 2012). The calcified cartilage is located between the deep zone of hyaline cartilage and the subchondral bone plate. Cells of the calcified cartilage are smaller compared to the deep zone (Buckwalter et al., 2005).

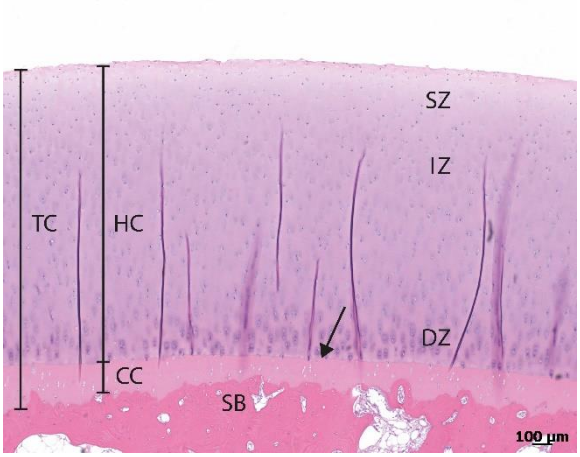


Figure 2: Histology of articular cartilage of a hematoxylin & eosin stained section

TC= *total cartilage*
HC= *hyaline cartilage*
CC= *calcified cartilage*
SB= *subchondral bone plate*
SZ= *superficial zone*
IZ= *intermediate zone*
DZ= *deep zone*
Arrow= *tidemark*

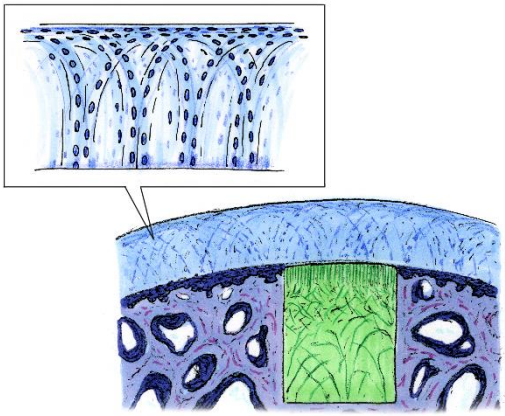


Figure 3: Appearance and orientation of collagen type II fibers and chondrocytes in articular cartilage of horses

2.3 Biochemical composition

Biochemically, articular cartilage consists of two main components: chondrocytes and the extracellular matrix (ECM). Chondrocytes located in the articular cartilage account for approximately only 1-12% of the total volume, while the rest is filled with ECM (Kuettner, 1992). All components of the ECM are produced by the chondrocytes. Density, morphology and metabolism of the chondrocytes varies within the different zones (Frisbie 2012).

The ECM is made up of three main components: water, collagen fibers (primarily collagen type II) and proteoglycans (PGs). Based on wet weight, water is considered the main component with an amount of 70% (ranges from 65-75%) (Frisbie 2012; Kuettner, 1992; Vachon et al., 1990). While calculated on dry weight, the main constituents are collagen fibers, which make up 60% of the dry weight and PGs make up around 30% of the dry weight. Further there is a smaller number of glycoproteins and lipids which together make up 10% of the dry weight (Vachon et al., 1990). Further there is also a small amount of non-collagenous proteins. These proteins contribute to organization and stabilization of the collagenous meshwork, and some also help chondrocytes to connect on macromolecules of the ECM (Buckwalter et al., 2005).

2.3.1 Collagens

With about 90-95%, collagen type II fibers are the primary found type of collagen in the articular cartilage of horses as well as in other mammals (Frisbie 2012). Other types of collagen represent only a small amount of the articular cartilage (Kuettner, 1992). These types are associated with the collagen type II fibers and are thought to provide stability to the collagen type II framework (Frisbie 2012).

Collagen type II fibers are secreted into the extracellular space as propeptides. After being released into the extracellular space, procollagen peptidases remove the excess endings (Clyne, 1987). Mature collagen type II fibers form fibrils, which are composed of 3 identical alpha I (II)-chains, organised in an alpha helix (Kuettner, 1992; Martel-Pelletier et al., 2008). Further these fibrils are organised in a 3-dimensional framework, which accounts for the tensile strength of the articular cartilage (Kuettner, 1992). The resistance of the articular cartilage to tensile and shear forces is most pronounced in the superficial cartilage layer, where the fibers are oriented parallel to the cartilage surface (Kuettner, 1992). Another important factor regarding the magnitude of resistance to tensile strength is the amount of crosslinks between the molecules. Studies have shown that a decreased number of crosslinks in turn led to a decline of the articular

cartilages` tensile property (McIlwraith 2011). The predominant crosslinks are 3-hydroxypyridinium crosslinks (Kuettner, 1992). As previously mentioned the greatest amount of collagen fibers are located in the superficial zone. Further these fibers posess smaller diameters compared to fibers located in deeper zones. There are small pores between the fibers of the superfical cartilage layer (diameter 6 nm). These pores allow small molecules such as glucose and ions to pass through, while large proteins such as hyaluronan cannot penetrate (Frisbie 2012). In contrast to the superficial zone, the fibers in the deep zone are oriented randomly and the pore diameter between the collagen fibers is increased to 40-100 nm (Frisbie 2012).

2.3.2 Proteoglycans

The other main components that make up the ECM are the PGs (Frisbie 2012). The content of PGs regulate the stiffness of the cartilage, and therefore provides resistance to compressive forces (Frisbie 2012). In contrast to collagen fibers, the biggest amount of PGs can be found in the deep zone and its content decreases towards the surface (McIlwraith 2011).

PGs consist of a protein backbone on which glycosaminoglycans (GAG's) are attached (Arokoski et al., 2000). PGs are enclosed into the complex framework of the collagen fibers. PGs can be divided into aggregated and non-aggregated PGs (Martel-Pelletier et al., 2008). The prevailing type of PGs (about 85%) consists of aggregated molecules named "aggrecans", which are further joined to large aggregates (McIlwraith 2011), consisting of about 100 solitary aggrecan molecules (Martel-Pelletier et al., 2008).

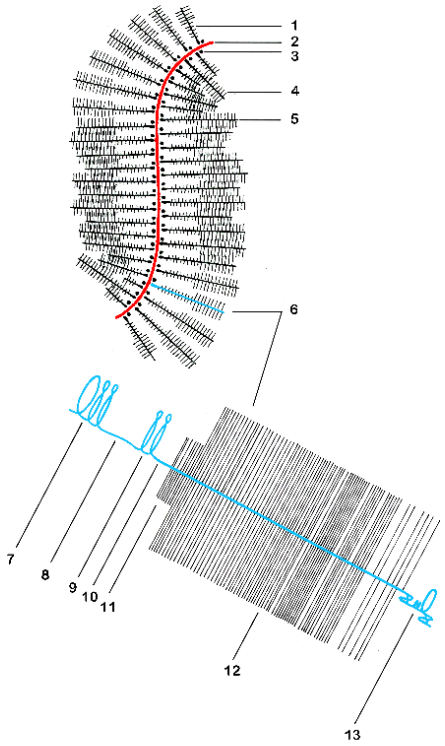
2.3.2.1 Aggrecan- an aggregated proteoglycan

A single aggrecan molecule is composed of a core protein, considered the backbone. The other main components, the GAG`s, are radially attached to the core protein. Chondroitin-6-sulfate, chondroitin-4-sulfate and keratansulfate are considered the main GAGs found in equine articular cartilage (Frisbie 2012, 1999; Clyne, 1987; McIlwraith 2011). In mature articular cartilage chondroitin-6-sulfate and keratansulfate are the predominant types. In immature articular cartilage chondroitin-4-sulfate is the major type and its amount decreases during maturation. Similar to other proteins, the core protein consists of an amino terminal (NH3) on one end and a carboxy terminal ($COOH^-$) on the other end. The carboxy terminal is a chondroitin rich region, in which around 100 chondroitin sulfate molecules are attached. On the other end the amino terminal is a keratansulfate rich region. The amino terminal is also named hyaluronic acid binding end, because its G1 domain binds to hyaluronan. This bond is further stabilized by a link protein (Frisbie 2012; Clyne, 1987).

The backbone can be further divided into different domains with different functions. Two core domains called G1 and G2, are located on the amino terminal of the protein (McIlwraith 2011). The G2 region, positioned on the aminoterminal end as well, operates as a cleavage site for enzymes which are involved in the breakdown of aggrecan (Frisbie 2012). The G3 domain, in contrast, is located at the carboxy terminal (McIlwraith 2011). This domain cannot be found in each aggrecan molecule, which thus may imply a less important role (Figure 4) (Frisbie 2012).

The GAG itself is constituted of repeating units of disaccharides. They are negatively charged because of their sulfated and carboxylated radicals in chondroitin sulfate and sulfated radicals in keratansulfate. The charges of the polyanionic side chains, tend to repeal each other and attract water (McIlwraith 2011). Consequently, they create a positive swelling pressure (Frisbie 2012) and deliver the typical stiffness to the articular cartilage (McIlwraith 2011).

Figure 4: Figure of an aggregate of about 100 aggrecan molecules and an enlargement of a single aggrecan molecule. Modified from Auer and Stick (Frisbie 2012).



Aggregate of about 100 aggrecan molecules

1) *Keratansulfate*
2) *Hyaluronan*
3) *Link protein*
4) *Chondroitin sulfate*
5) *Core protein of a single aggrecan*

A monomer of a single aggrecan molecule

6) *One single aggrecan molecule*
7) *G1 domain*
8) *IGD=interglobular domaine (region for proteolytic cleavage)*
9) *G2 domain (cleavage site for enzymes which are involved in breakdown of aggrecan)*
10) *Core protein*
11) *Keratansulfate region*
12) *Chondroitinsulfate region*
13) *G3 domain*

2.3.2.2 Non aggregated proteoglycans

The smaller number of PGs (about 5% of the total mass of PGs) are non-aggregating, small PGs (McIlwraith 2011). They are smaller, with a shorter backbone compared to the aggrecan molecules and thus called small PGs (Frisbie 2012; Buckwalter et al., 2005). Similar to aggrecan they consist of a core protein with covalently attached GAGs (Frisbie 2012). Examples of non-aggregated small proteoglycans are biglycan, decorin and fibromodulin (Martel-Pelletier et al., 2008; Frisbie 2012). Decorin consists of one dermatan sulfate chain, biglycan is composed of two dermatan sulfate chains and fibromodulin consists of more than a few keratansulfate chains. Presumably more types of non-aggregated PGs exist, but to date they have not been described. In contrast to aggrecan, these non-aggregating, small PGs build only a small portion of the total volume of PGs and therefore do not influence the mechanical properties of the articular cartilage. Nevertheless, fibromodulin and decorin are attached to collagen type II fibrils and thus might contribute to the stability of the collagen network. Small PGs are furthermore capable to bind transforming growth factor-β (TGF-β) and consequently are thought to have influence on its activity (Buckwalter et al., 2005).

2.4 Articular cartilage – metabolism

Similar to other tissues, articular cartilage has a turnover in which catabolic and anabolic processes are kept in balance (Frisbie 2012). It is worth mentioning, that the metabolic activity of the articular cartilage is much slower compared to other tissues. In cartilage half-life of PG is much shorter compared to collagen type II fibers (Kuettner, 1992). The metabolism of articular cartilage is mainly controlled by different growth factors. They have the ability to stimulate chondrocytes to synthesize all ECM components. Although named growth factors, some of them do not only promote growth, in contrast they may also promote degradation of the articular cartilage constitutes and therefore play an important role in the pathogenesis of OA. One of the most important growth factors is transforming growth factor β (TGF-β). The effects of different growth factors (TGF and insulin dependent growth factor (IGF)) on articular cartilage metabolism are shown in table 1. TGF-β mainly stimulates chondrocytes to proliferate and consequently produces the major ECM components. Further TGF-β is able to inhibit metalloproteinase 9 and 1 (MMP-9 and MMP-1), which are degradative enzymes, and therefore provide a protective function to the cartilage. TGF-β also interacts with interleukin-1β (IL-1β) neutralizing its suppressive action on the PG metabolism. On the other hand it also has the potential to stimulate MMP-13 and the aggrecanase "a disintegrin and MMP with thrombospondin motifs" (ADAMTS-4) thus leading to articular cartilage degradation. Another important growth factor is IGF. This factor is a polypeptide, which is essential in stimulating growth of many different tissues. In articular cartilage it stimulates chondrocyte proliferation and thus stimulates the synthesis of the major cartilage matrix components. In healthy cartilage this factor is only expressed at low levels, but in the case of OA its concentration increases. IGF only up regulates the production of PGs without having a negative regulatory mechanism for cartilage health, unlike TGF-β, which can act both ways (Martel-Pelletier et al., 2008).

***Table 1**: Effects of growth factors on articular cartilage metabolism*

TGF- β	**IGF**
positive effects: *Stimulates chondrocytes to proliferate and produce ECM *Inhibits MMP- 1 and MMP 9 *Inhibits IL-1β	**positive effects:** *Stimulates chondrocytes to proliferate and produce ECM
negative effects: *Stimulates MMP-13 and ADAMTS	**negative effects** *none

2.5 Osteoarthritis

2.5.1 Osteoarthritis in general

The term osteoarthritis (OA) relates to the disease of the articular cartilage and is further characterized by degeneration and destruction of the articular cartilage (Frisbie 2012). Other common terms often used to describe this disorder include degenerative joint disease or osteoarthrosis. The most favored term is OA (Frisbie 2012).

2.5.2 Osteoarthritis in clinical patients

OA is one of the most common reasons for lameness (60%) (McIlwraith et al., 2012) and therefore has an economic relevance. OA in the metacarpophalangeal and the carpal joints mostly affects racehorses and forces an early ending of their career (McIlwraith et al., 2012). OA of the distal interphalangeal joint is a common orthopedic disease in horses (Dyson, 1991; Kristiansen and Kold, 2007; McKnight and Posh, 2012). OA causes lameness of varying degrees (Kidd et al., 2001; Trotter and McIlwraith, 1996). In some cases, horses will only show lameness or stiffness for the first few steps after being taken out of the stall. During palpation of the affected joint, swelling, pain and heat are commonly found local clinical signs. Further, joint flexion may be painful. Anesthesia of the palmar digital nerves and intraarticular anesthesia of the distal interphalangeal joint are useful to identify the source of pain (Dyson, 2010; Trotter and McIlwraith, 1996). Thereafter, diagnostic imaging, such as radiographs and ultrasound should be performed to identify morphological changes. In more difficult cases, before abnormalities can be seen on radiographs, MRI may be needed to identify any underlying disease. MRI enables a definitive diagnosis of early OA in horses, even if no radiographic changes can be seen (Dyson, 2010).

Because of the vast economic relevance, both prevention and adequate treatment in a timely manner are important (Trotter and McIlwraith, 1996). Although improvement in treating OA with newly invented strategies during the last decades has taken place, a satisfying therapy has not been developed yet. Furthermore, due to the absence of an appropriate natural repair mechanism of articular cartilage, healing of this tissue still remains a challenge. Even if spontaneous repair mechanisms exist, they are not satisfying, because the characteristics of the newly built cartilage resembles fibrous tissue, rather than hyaline cartilage. As a consequence the biochemical and biomechanical properties of the repair tissue are reduced and the newly built cartilage is more susceptible to injuries (Wilke et al., 2007). Thus the main objective

remains early and appropriate diagnosis allowing timeous initiation of therapy to prevent further progression of disease (Trotter and McIlwraith, 1996)

2.5.3 Pathogenesis of osteoarthritis

The abnormalities occuring in osteoarthritic joints include various stages of degeneration of articular cartilage, fibrosis of the joint capsule, thickening of the synovial membrane, synovitis, depolymerisation of hyaluronic acid, osteophyte formation and sclerosis of the subchondral bone (Frisbie 2012).

Numerous theories for the pathogenesis of OA exist (Frisbie 2012). As a conlusion there are two main principles for the development of OA; either a normal articular cartilage which has to deal with abnormal forces or an abnormal cartilage that cannot resist the physiological forces (McIlwraith et al., 2012). Further it is not clarified whether the articular cartilage or the subchondral bone is the primarily involved structure.

If the changes occur in the articular cartilage first, the altered composition of articular cartilage leads to different biochemical properties, thus leading to cartilage less resistant to compressive and tensile forces. For example in humans, a genetic failure, which leads to abnormal type II collagen has been reported. Trauma due to use is considered a common cause for the development of OA. Although joint loading is essential for enabeling lubrication and furthermore activates the production of PGs, loads above a specific level and lead to damage of the articular cartilage. At such high loads, the chondrocytes are not able to produce enough PGs to resist the increased loading and failure of the articular cartilage may result (Kidd et al., 2001). Another theory assumes that the subchondral bone is the primary problem. Physiologically the density of the subchondral bone increases under load. If bone densitiy increases to much and reaches a specific threshold, the subchondral bone is less compliant, leading to damage of the articular cartilage. Another important component for the development of OA is synovitis. This inflammation process releases various cytokines (IL-1β, tumour necrosis factor alpha (TNF-α)), degradative enzymes (MMP's and aggrecanases) and also inflammatory mediators (prostaglandins) (Kidd et al., 2001).
In summary, most of joints composites are involved in the pathogenesis of OA, and therefore altered in such a joint (Frisbie 2012).

2.5.3.1 Role of cytokines

IL-1 β

As mentioned above, cytokines play a fundamental role in the pathogenesis of OA. The cytokine which is primarily upregulated in OA is IL-1β (Martel-Pelletier et al., 2008). IL-1β has various pathogenic properties. It upregulates the production of MMP's, inhibits the synthesis of their natural inhibitors and further prevents the synthesis of the major ECM components, such as collagens and PGs. As a result the typical damage of articular cartilage occurs. Moreover IL-1β activates fibroblasts to secrete type I and III collagen, resulting in fibrosis of the joint capsule observed in chronically inflammed joints (Kidd et al., 2001).

TNF-α and release of MMP's

Another cytokine which is central in the pathogenesis of OA is TNF-α. TNF-α leads to the upregulation of MMP's. Those degradative enzymes are capable of damaging the major components of the articular cartilage (Kidd et al., 2001). MMP's are degradative enzymes which require zinc at their active binding site. Both chondrocytes and synoviocytes are able to release MMP's (Frisbie 2012). MMP's are secreted as inactive proenzyms, which require activation by serine proteinases (McIlwraith 2011). The different MMP's as a collective are able to degrade every single component of the articular cartilage. Noteable is, that areas which showed histological damage, also revealed increased levels of MMP's. This implies the relevance of MMP's in the degradation of articular cartilage (Frisbie 2012). MMP's can be further divided into three different subtypes (McIlwraith 2011), according to their first detected substrate (Frisbie 2012). The different substrates of the MMP`s are shown in table 2. The three different subtypes include **collagenases**, **stromelysins** and **gelatinases** (McIlwraith 2011). Of the enzyme collagenase three different forms have been isolated; named MMP-1, MMP-8 and MMP- 13 (Martel-Pelletier et al., 2008). MMP-13 is most commonly involved in degradation of collagen type 2 fibers in horses and humans. This MMP is secreted by equine chondrocytes and the secretion is stimulated by recombinant human interleukin 1 (rhIL-1) (McIlwraith 2011). Collagenase MMP-1 has also been shown to break down collagen type II fibers (Frisbie 2012). Stromelysins are MMP's which have the capability to degrade PGs as well as fibronectin, elastin and laminin. To date three different types of stromelysins have been detected; MMP-3, MMP-10 and MMP-11. MMP-3 can be considered as the major stromelysin, involved in cartilage matrix turnover and was found at increased levels in cartilage showing early signs of

OA. There are 2 different types of gelatinases: MMP-9 and MMP-2. Their substrates include denatured collagen type II, gelatin and collagen type V and VI (Martel-Pelletier et al., 2008)

ADAMTS and TIMPS

MMP's are not the only enzymes which are able to breakdown the ECM. There are additional enzyms possessing the ability to degrade the ECM components called "aggrecanases". These enzymes are therefore able to degrade the molecule "aggrecan". Another name for these enzymes is ADAMTS-4 and 11 (McIlwraith 2011).

For each MMP's natural inhibitors exist, which are called "tissue inhibitor of metalloproteinases" (TIMP). There are two different subtypes; TIMP-1 and TIMP-2. The balance of TIMPs and MMPs is important in the light of articular cartilage degradation. In osteoarthritic joints, decreased a level of TIMPs could have been detected (Kidd et al., 2001).

***Table 2**: Substrates of the different MMP's*

Collagenases	
MMP-13 MMP-8 MMP-1	collage type II fibers
Gelatinases	
MMP-9 MMP-2	denaturated collagen type II gelatin collagen type VI und V
Stromelysins	
MMP-3 MMP-10 MMP-11	all types of PGs

2.6 Macroscopic changes in osteoarthritic cartilage

Various methods exist for the macroscopical grading of OA. A frequently used system for the macroscopical assessment of a joint, are the criteria of Outerbridge (Table 3). It was first developed by Outerbridge for the grading of chondromalacia of the patella in humans (Outerbridge, 1961). Fibrillation of the superficial cartilage is considered the first visible sign of OA. Later fissures and erosions occur (Buckwalter et al., 2005). Assessed criteria according to McIlwraith include wear lines and partial- or full-thickness erosion. Wear lines are fissures which can be seen in cartilage with mild OA. Wear lines are findings present in horses with early OA and are thought to reduce the mechanical properties of articular cartilage (McIlwraith et al., 2010).

Besides there are also techniques to grade spontaneous OA using Indian ink stain to detect disease of the articular cartilage. It is a stain, which normally cannot enter intact articular cartilage. In cases of OA, with increased depletion of PGs the stain is able to infiltrate the cartilage and stain uptake is visible. Nevertheless, grading of the articular cartilage is usually done without Indian ink (McIlwraith et al., 2010).

2.7 Histological changes in osteoarthritic cartilage

The surface of a healthy articular cartilage appears smooth and constant under light microscopy. In early stages of OA, surface irregularities and fibrillation of the superficial zone occur. Further PG content decreases and results in loss of staining of the superficial zone in Safranin - O - Fast Green (SOFG) or toluidine blue (TB) stained sections. In further stages, fissures of the superficial zone arise and the stain loss expands into deeper zones. As OA progresses erosions with destruction of the cartilage surface may occur. Primarily only the superficial zones are damaged. Later the erosions can expand up to the subchondral bone and are called "full thickness erosions". A further interesting point is the integrity of the tidemark. In some cases with OA, a breakthrough of blood vessels through the tidemark into the cartilage can be detected (Buckwalter et al., 2005).

Cartilage sections with OA can be scored using different grading systems. To date there are two mainly used scoring systems (Aigner et al., 2010). A widely used grading system is the histological histochemical grading system (HHGS) system developed by Mankin et al in 1971. The original Mankin score was developed to grade human femoral heads with OA, stained with Safranin – O light green (Mankin et al. 1971; Pritzker et al., 2006). Original criteria include structure of the cartilage surface (normal, surface irregularities, clefts into different zones), density of chondrocytes (normal, diffuse hypercellularity, cloning, hypocellularity), the amount of staining with the Safranin-O-Fast Green (SOFG) (normal stain, slight reduction, moderate reduction, severe reduction, no dye noted) and the integrity of the tidemark (intact or crossed by blood vessels) (Mankin et al. 1971).

In 2006, a new invented scoring system called the Pritzker Score system was initiated by an "osteoarthritis research society international" OARSI initiative in 2006 (Aigner et al., 2010; Pritzker et al., 2006). The aim was to standardize the evaluation of OA by inventing a simple and widespread grading system with high reproducibility and validity. Further it was developed because of the lack of a system for early stages of OA (Pritzker et al., 2006).

2.8 Biochemical changes in osteoarthritic cartilage

On a biochemical level, one of the first indications of OA is a decrease of the PG content and an increase of the water content in the ECM (Arokoski et al., 2000). Physiologically the collagen framework limits the amount of water that can be attracted by the negatively charged GAG's (Goodrich and Nixon, 2006). In early stages of OA, a disorganization of the collagen network occurs (Arokoski et al., 2000). This allows the GAG's to attract more water and the water content increases (Kidd et al., 2001). In a later stage of the disease, fibrillation as well as breakdown of the collagenous framework arises (Arokoski et al., 2000). This breakdown of the collagen fibrils can be detected by biomarkers, even if no morphological abnormalities can be seen yet (McIlwraith 2011). In the early stages, the destruction of the collagen fibrils happens solely in the superficial zone of the cartilage (Arokoski et al., 2000). If superficial erosions occur, the deeper layers are also more susceptible to damage because they are less resistant to the physiological forces (McIlwraith 2011).

2.8.1 Biochemical distribution pattern within the joint

To date differences of GAG and water content in the ECM of fetlock and carpal joints have been detected (Brama et al., 2000; Murray et al., 2001; Murray et al., 1999). The fetlock joint is a widely examined joint in racehorses because it is one of the joints primarily affected by OA. A study of Brama et al. 2000 revealed regional differences in PG, water content and collagen type II content in the healthy fetlock joint. Further each parameter has shown a typical topographical distribution pattern. P1 had shown significant higher water and deeper GAG content in mediodorsal and laterodorsal regions, compared to region of interest (ROIs) in central areas. Similar findings were seen in the MC bone, where ROIs located in dorsal regions had shown a significant higher water content, and lower GAG content compared to central located regions (also centrodorsal). Also, two samples (medial and lateral) from the proximal sesamoid bone were taken, which showed no relevant differences in GAG and water content (Brama et al., 2000).

Another joint often affected by osteochondral lesions in racehorses is the middle carpal joint. Murray et al. had analyzed the water, GAG, DNA and hydroxyprolin content from the intermediate, radial and third carpal bone. The study revealed significant differences in GAG content between dorsal and palmar sides. Water content has shown no differences between the sites. GAG content was significantly higher in palmar regions compared to dorsal sides. Lesions are mostly observed at the dorsal margin of the middle carpal joint (Murray et al., 2001) and on the dorsal aspect of third carpal bone in racehorses (Lacourt et al., 2012).

2.8.2 Aims of the study

Despite the relatively high prevalence of OA in the distal interphalangeal joint, especially in Warmbloods, there's a lack of information about the biochemical properties of this particular joint to date. Therefore, the aims of this study are:

- To describe the distribution of the major ECM components (PG, water, collagen structure) in the normal cadaver distal interphalangeal joint at different regions of interest (ROIs)
- To identify the topographical variations of the ECM components (PG, water, collagen structure) within the normal cadaver equine distal interphalangeal joint.
- To identify changes of the ECM components (PG, water, collagen structure) in normal compared to osteoarthritic cadaver distal interphalangeal joints.

We hypothesized that:

- There will be topographical variations in the composition of the ECM within the normal distal interphalangeal joint.
- The ECM (PG, water, collagen structure) will change significantly between normal and osteoarthritic joints.

3. Material and methods

3.1 Horses and macroscopical cartilage examination

Samples were collected from the distal interphalangeal joint of 12 cadaver forelimbs (5 right limbs, 7 left limbs). Limbs were obtained from 12 different horses with a mean age of 15.17 ± 9.2 (6-32) years (9 males and 3 females). Horses were euthanized for diseases unrelated to the distal interphalangeal joints. The distal interphalangeal joints were dissected and opened directly after euthanasia. First they were inspected macroscopically if any lesions of articular cartilage were visible (Figure 5).

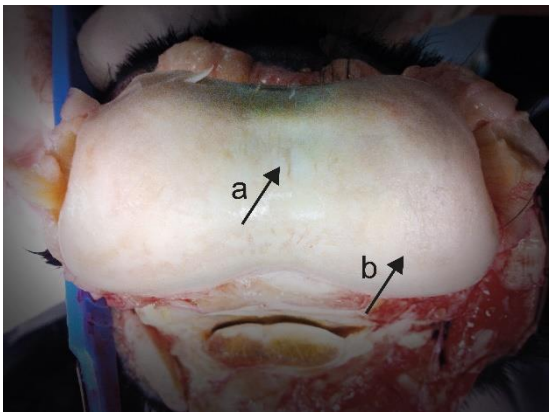


Figure 5: Surface of P2 of a cadaver distal interphalangeal joint

a) *Partial thickness fissure*

b) *Partial thickness erosion of the surface of articular cartilage*

3.2 Collection of samples

Samples from different ROI's as shown in Figure 6 and 7, were collected from each joint. Six from P2 (ROIs 1-3 and 7-9), and five from the P3 (ROI 11, 13, 14, 15, 17). Nine mm diameter osteochondral samples were taken using an Osteochondral Autograft Transfer System (allograft OATS® (Arthrex Swiss AG, Bern-Belp, Switzerland)). A central section was cut from the osteochondral plug and the section was immediately processed for the histological examination. The cartilage was transected from the subchondral bone using a scalpel. It was paid attention to obtain full thickness samples, which included the calcified cartilage but excluded the subchondral bone. Cartilage samples were stored at -80° for further biochemical analysis.

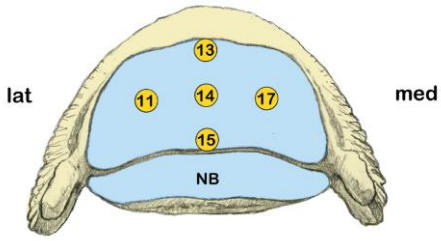


Figure 6:
ROIs harvested from the proximal aspect of P3.

***NB**= Navicular bone*

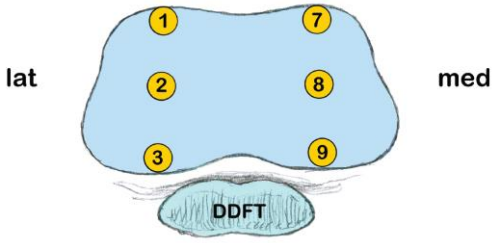


Figure 7:
ROIs harvested from the distal aspect of P2.

***DDFT**= Deep digital flexor tendon*

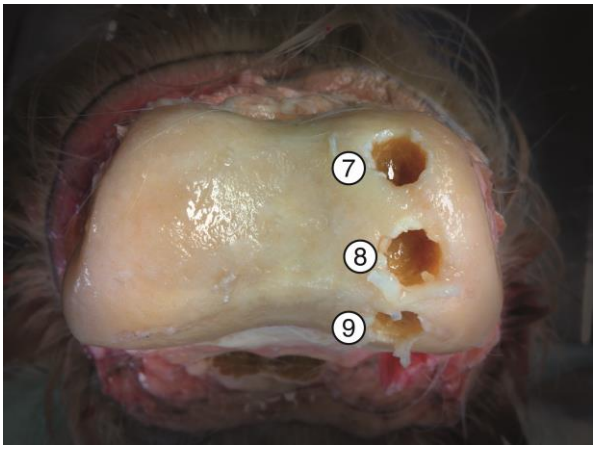


Figure 8:
Examples for full thickness biopsies at ROI 7, 8 and 9

3.3 Water content

The cartilage samples were weighed after defrosting to obtain their weight using a XP 105 DeltaRange® analyze scale (Mettler-Toledo, Greifensee, Switzerland). Following weighing the samples were lyophilized, using a Speed Vac® plus machine (Labexchange, Burladingen, Germany) for four hours. The lyophilized samples were weighed again, their dry weight was recorded and samples were stored at -80° for further analysis. The total amount of water was calculated using the formula: [(wet weight - dry weight)/ wet weight]. Water content was expressed as % of wet weight.

3.4 Determination of glycosaminoglycans

Determination of GAG concentration was performed in collaboration with the TETEC© laboratory in Reutlingen, Germany. Biochemical analysis were performed following a modified protocol of the dimethylmethylenblue (DMMB) assay first developed by Farndale et al. (Farndale et al., 1986). This method is based on the principle that sulfated GAGs of the ECM build a complex with the DMMB dye solution. The absorbance of these complexes can be measured in a spectrometer at a specific wavelength. Thus the concentration of sulfated GAGs can be calculated.

The dried samples were digested overnight with an appropriate volume of papain at 60° to minimalize the interference by other proteins or glycoproteins.

For further measurements a papain buffer was prepared which contained sodium acetate (W302406, Sigma-Aldrich, Buchs), sodiumchloride (71380, Sigma-Aldrich, Buchs) L-cysteinhydrochlorid, and sodium-ethylenediaminetetraacetic acid. The buffer was set at a PH 6.0 and stored at room temperature. Further a DMMB stock solution was prepared containing: 1,9 dimethylmethylenblue (341088, Sigma-Aldrich, Buchs, Switzerland), sodium formate (17841, Sigma-Aldrich, Buchs, Switzerland), ethanol (51976, Sigma-Aldrich, Buchs, Switzerland) and formic acid (Sigma-Aldrich, Buchs, Switzerland), and was set at pH 3.0. The solution was stored at room temperature in a brown bottle to prevent the solution from light (Farndale et al., 1986).

The absorbance was measured at a wavelength of 520 nm with a PHERAstar©- microplate reader (Labtech® GmbH, Ortenberg, Germany).

The dried and papain digested samples were diluted appropriately and then placed as triplicates into the 96 well plates (flat bottom) (Sigma-Aldrich, Buchs, Switzerland). Shark chondroitin sulfate (C-4384, Sigma-Aldrich, Buchs, Switzerland) was used as a standard to create the standard curve. Chondroitin sulfate was placed in a concentration of 0, 2.5, 5, 10, 20, 40, 80, 100, 150 and 200µg/ml into the plates. Samples, papain buffer or standards had been placed in triplicates of 14µl into a 96 well plate (Farndale et al., 1986). To determinate the concentration of sulphated GAG, 266µl of the prepared DMB solution was added to each well and the absorbance was measured at a wavelength of 520 nm. Absorbance was measured immediately after adding the DMB solution, because of the aggregation of the sulfated GAG with the DMB dye. Further care was taken not to mix the plate after the dye was added, to prevent enhanced precipitation. Number of GAG's were calculated using the standard curve and results were expressed as µg/mg cartilage normalized on wet weight.

3.4 Histological analysis

3.4.1 Preparation of histological sections

For the histological evaluation, cartilage samples were fixed in 4% paraformaldehyde for about 48 hours, were decalcified in 25% EDTA for approximately 4 weeks and were put in paraffin. Sections were cut and stained with haemotoxylin and eosin (HE), SOFG, TB and picrosirius red (PR). The histological sections were scored using a modified Mankin scoring system on SOFG stained sections (Table 3). Each ROI was scored separately by three blinded observers. Later the scores of each criterion were added and a total score was determined. Sections were divided into three different grades of OA: normal cartilage (Mankin score 0-1.9), mild to moderate OA (Mankin score 2.0-8.0), and severe OA (8.1-16.0) (Lacourt et al., 2012).

Table 3: *Modified Mankin scoring system developed by Lacourt et al. 2012*

Structure	
0	Normal
1	Fibrillation
2	Fissures
3	Erosion of 1/3 of depth of the hyaline cartilage
4	Erosion of 2/3 of depth of the hyaline cartilage
5	Full depth erosion of the hyaline cartilage
6	Full depth erosion of the hyaline and calcified cartilage
Chondrocyte density	
0	No decrease in cells
1	Focal decrease in cells
2	Multifocal decrease in cells
3	Multifocal confluent decrease in cells
4	Diffuse decrease in cells
Cluster formation	
0	Normal
1	< 4 clusters
2	≥ 4 but < 8 clusters
3	≥ 8 clusters
Safranin O - Fast Green	
0	Uniform staining throughout articular cartilage
1	Loss of staining in the superficial zone of hyaline cartilage
2	Loss of staining in the upper 2/3 of hyaline cartilage
3	Loss of staining in all of hyaline cartilage

3.4.2 Proteoglycan content on Safranin-O-Fast green and toluidine blue stained sections

In this study, the content of PGs was quantified both on SOFG and TB stained slices. The amount of decrease of PGs was estimated according to criteria of table 3. The amount of stainloss was part of the assessment criteria using a modified Mankin score (Table 3). Physiologically a uniform staining throughout the whole cartilage is visible. In horses with OA, loss of the PG content leads to stain loss. First stain loss occurs only in the upper 1/3. In later stages the stain loss expands up to 2/3 and in severe cases stain loss in all layers of articular cartilage is visible.

3.5 Assessment of collagen type II

3.5.1 Picrosirius red

Samples were stained with the PR dye. In PR stained sections orientation of collagen type II fibers in the different zones was analyzed on a polarized microscope with a specific filter. The assessment in this study was done with a Leica DMI 6000 microscope with a polarizing filter at the center for microscopy and image analysis at the University of Zurich, Switzerland.

Slices were divided in four groups as shown in table 5: normal articular cartilage, samples with loss of integrity of collagen type II fibers in about 1/3 of cartilage thickness, samples with loss of 2/3 of integrity of cartilage thickness and samples with loss of integrity of 3/3 of articular cartilage (Table 6).

***Table 4**: Collagen structural grade on Picrosirius Red stained samples*

Severity score	
0	Normal
1	Loss of integrity of 1/3 cartilage thickness
2	Loss of integrity of 2/3 cartilage thickness
3	Loss of integrity of 3/3 cartilage thickness

3.5.2 Immunohistochemistry of denaturated type II collagen (=collagen type II cleavage products)

Determination of collagen type II cleavage products was done with Immunohistochemistry. Sections were processed according to the attached protocol (supplements). Briefly sections were dewaxed, incubated and digested with hyaluronidase at a temperature of 37°. After this, sections were washed with phosphate buffered saline (PBS). Sections were set at 37° and were incubated with the primary antibody (collagen type II rabbit antibody ab34712, Abcam Inc, Cambridge, MA, USA) with a dilution of 1:100. After incubation with the primary antibody slices were washed in PBS again to remove the antibodies which did not bind with the epitope (denaturated collagen type II= cleavage product of collagen type II fibers). After the washing step, a secondary antibody (goat antibody against rabbits 1:100, Thermo scientifics, Illinois, USA) was used in order to make cleavage products visible.

To classify the amount of denaturated collagen type II, the dimension of stain was assessed according to the following table. First stain uptake was evaluated. Further extension into the surface was accessed as "stain location". The degree of stain was evaluated to quantify the number of collagen type II cleavage products (table 6). Stain was compared to the staining of a healthy articular cartilage surface.

***Table 5**: Grading of collagen type II on immunohistochemistry*

Stain uptake	
0	No staining
1	Focal staining
2	Multifocal staining
3	Multifocal confluent staining
4	Diffuse staining
Stain location	
0	No staining
1	Staining in upper 1/3 of hyaline cartilage
2	Staining in upper 2/3 of hyaline cartilage
3	Staining in all hyaline cartilage
Stain degree	
0	No staining
1	Light staining
2	Intermediate staining
3	Dark staining

3.6 Data analysis

Statistical analysis was performed with the SPSS® software version 21.0 (SPSS INC, Chicago, USA). ROIs were divided into different groups depending on their location within the distal interphalangeal joint for further statistical evaluation. Horizontal plane, (dorsal (ROIs 13, 1, 7) /middle (ROIs 11, 14, 17, 2, 8)/palmar (ROIs 15, 3, 9)), sagittal plane (condylar (ROIs 11, 17, 1, 2, 3, 7, 8, 9) /groove (ROIs 13, 14, 15)) and a further subdivision was done between P2 (ROIs 1, 2, 3, 7, 8, 9) and P3 (ROIs 11, 13, 14, 15, 17).

Distribution of data was calculated with a Kolmogorov-Smirnoff test of normality. Results of parametric data were exposed as mean ± standard deviation, while results of nonparametric data were exposed as median (range). To verify the inter observer reliability an intra-class correlation coefficient (ICC) was determined for the various histological scores (overall mean modified Mankin score, overall mean immunohistochemical score and the overall mean PR score). An ICC >0.7 was rated as good, an ICC >0.8 as optimal and an ICC >0.9 as excellent. Two generalized linear models with a gamma log link were performed. One with water content as the dependent variable and state of OA (normal articular cartilage/mild to moderate OA/severe OA), horizontal plane (dorsal/ middle/ palmar) and sagittal plane (condyles/groove) as independent variables. The other generalized linear model was performed with GAG content as the dependent variable and the same independent variables as above. Statistical associations between the overall mean Mankin score, the overall mean PR score, the overall mean immunohistochemistry score, the mean water content and the mean GAG content (wet weight) were calculated with Spearman`s non-parametric correlations. Results were rated as significant if $P < 0.05$.

4. Results

4.1 Glycosaminoglycan and water content

GAG (normalized on ww) and water content of the different regions of the distal interphalangeal joint divided by health status (normal, mild to moderate OA, severe OA) are shown in Table 7-9.

Table 6: *GAG and water content of different cartilage specimens of the distal interphalangeal joint*

Parameters	**Water %**	**N=**	**GAG ww (µg/mg)**	**N=**
normal cartilage	71.0 (59.2-83.0)	40	60.0 (21.0-104.2)	40
mild- moderate OA	73.0 (47.9-82.0)	66	49.7 (17.5-106.0)	66
severe OA	76.5 (64.0-83.1)	14	30.4 (15.0-73.2)	14
dorsal	72.0 (47.9-82.0)	36	55.03 (17.5-106.0)	36
middle	74.1 (54.3-83.0)	53	46.2 (19.0-99.8)	53
palmar	69.9 (63.3-83.1)	31	53.5 (15.0-96.7)	31
condyle	70.8 (47.9-83.1)	88	53.4 (15.00-106.0)	88
groove	74.1 (67.1-83.0)	32	47.5 (18.4-65.2)	32
P2	69.6 (47.9-83.1)	68	60.0 (15.0-106.0	68
P3	74.5 (54.3-83.0)	52	45.9 (18.38-80.8)	52

GAG (normalized on wet weight (ww)) and water content of the different regions of the distal interphalangeal joint divided by health status (normal, mild to moderate OA, severe OA) (Table 7-9).

Table 7: *GAG and water content of healthy distal interphalangeal joint cartilage*

Parameters	**Water (%)**	**N=**	**GAG ww (µg/mg)**	**N=**
condyle	69,1 (59,2-74,8)	28	64,00 (42,1-104,2)	28
groove	73,5 (67,1-83,0)	12	48,7 (21,0-65,2)	12
dorsal	71,7 (59,2-78,8)	14	59,0 (36.37-104,1)	14
middle	71,5 (63,8-83,0)	17	60,7 (21,0-99,8)	17
palmar	68,3 (63,3-78,1)	9	65,5 (42,1-96,8)	9

Table 8*: GAG and water content of distal interphalangeal joints with mild to moderate OA*

Parameters	**Water (%)**	**N=**	**GAG ww (µg/mg)**	**N=**
condyle	71,9 (47,9-82,0)	49	50,0 (17,5-106)	49
groove	74,1 (69,4-81,6)	17	49,3 (18,3-64,9)	17
dorsal	72,7 (47,9-82,0)	21	51,0 (17,5-106,0)	21
middle	76,2 (54,3-81,6)	28	44,4 (23,4-74,6)	28
palmar	69,9 (64,0-77,2)	17	53,5 (38,4-79,7)	17

***Table 9**: GAG and water content of distal interphalangeal joints with severe OA*

Parameters	**Water (%)**	**N=**	**GAG ww (mg/mg)**	**N=**
condylus	75,5 (64,0 83,1)	11	31,7 (15,0-73,2)	11
groove	77,5 (73,1-78,2)	3	29,1 (26,8-43,5)	3
dorsal	64,9 (64,9-64,9)	1	73,2 (73,2-73,2)	1
middle	78,0 (64,0-79,9)	8	29,3 (19,0-69,2)	8
palmar	75,5 (64,3-83,1)	5	29,1 (15,0-62,0)	5

Tables 10 and 11 show the mean water content in %, the mean GAG content in (µg/mg), the 95% confidence intervals (CI) and P-values of the generalized linear models from the different grades of OA, as well as from different locations within the DIPJ.

***Table 10:** Generalized lineal models with water (in %) as dependent variable. Mean, confidence interval (CI) and P-value for the different independent parameters are listed.*

	Water	**(%)**	
Independent parameters	mean	95% CI	P-value
normal cartilage	70.8	69.9-71.8	<0.0001
mild to moderate OA	73.1	72.3-73.9	0.046
severe OA	74.9	73.2-76.6	-
dorsal	72.0	70.8-73.1	0.842
middle	75.0	74.0-76.0	<0.0001
palmar	71.8	70.7-73.0	-
condyle	70.6	69.9-71.4	<.0001
groove	72.2	74.1-76.4	-

***Table 11**: generalized linear models with GAG (µg/mg) as dependent variable. Mean, confidence interval (CI) and P-value for the different independent parameters are listed.*

	GAG	**(µg/mg)**	
Independent parameters	mean	95% CI	P-value
normal cartilage	58.4	55.0-62.1	<0.001
mild to moderate OA	48.0	45.7-50.4	<0.0001
severe OA	37.2	33.6-41.1	-
dorsal	48.6	45.3-52.1	0.498
middle	42.7	40.2-45.3	<0.001
palmar	50.2	46.7-53.9	-
condyle	52.1	49.7-54.7	<0.001
groove	42.5	39.6-45.5	-

4.1.1 Regional differences of all grades of osteoarthritis in various regions

Water content in middle zones was 75%, 72% in dorsal zones and 71.8% in the palmar zones. Water content in middle zone was significantly higher compared to palmar zones ($P < 0.001$). Between water content of dorsal zones compared to palmar zones no significant difference was found ($P = 0.498$).

Mean GAG content in dorsal regions was 48.6 µg/mg, 42.7 µg/mg in middle areas and 50.2 µg/mg in samples located in palmar regions. Mean GAG of the middle zones was significantly lower compared to cartilage samples located in palmar zones ($P < 0.001$). Between GAG content of the dorsal zones and the palmar zones, no significant difference was observed ($P = 0.498$).

Further, ROIs from samples located in the condylar area had significantly deeper water content compared to samples located in the condylar groove ($P < 0.001$). GAG content was significantly higher in condylar regions compared to samples located in the condylar groove.

4.1.2 Severity of osteoarthritis

Water content of all locations was 70.8 % in normal cartilage samples, 73.1% in samples with signs of mild to moderate OA, and 74.9 % in samples with severe OA. Thus, water content in this study was significantly deeper in healthy cartilage samples compared to samples with severe OA ($P < 0.001$). Further, water content was lower in samples with mild to moderate OA, compared to samples with severe OA ($P = 0.046$).

Mean GAG content in healthy cartilage samples was 58.4 µg/mg, 48,0 µg/mg in samples with mild to moderate OA and 37.2 µg/mg in samples with severe OA. GAG content of healthy cartilage samples was significantly higher compared to samples of severe OA ($P < 0.001$). Mean GAG content of cartilage with mild to moderate OA was also significantly higher compared to samples with severe OA ($P < 0.001$).

The GAG content negatively correlated with the mean overall Mankin score (spearman's rho correlation coefficient (rs): - 0.394; $P < 0.001$), with the mean overall PR score (rs: -0.212, $P < 0.001$) and with the overall histochemical score (rs: -0.170; $P < 0.001$). A significant negative correlation was observed between the GAG and water content (rs: -0.773 $P < 0.001$).

Water content significantly correlated with the mean overall Mankin score (rs: 0.324, $P < 0.001$) and the mean PR score with (rs: 0.144, $P < 0.009$). Between the mean water content and the mean overall immunohistochemical score, no significant correlation was observed (rs: 0.096, $P = 0.077$).

4.2 Light microscopy

4.2.1 Safranin - O- Fast Green and Toluidine blue stained sections - Mankin scoring

A total of 120 Region of Interest (ROIs) were scored by three blinded observers. Forty sections were scored as healthy cartilage. Sixty-six sections were classified as mild to moderate OA and 14 were scored with severe OA. The ICC for the mean Mankin score of the three blinded observers was 0.98. Representative images of the different cartilage lesions are shown in Figure 9. There was a significant correlation between the mean overall Mankin score and the mean overall PR score (rs: 0.427, $P < 0.001$) and the mean overall immunohistochemical score (rs: 0.610, $P < 0.001$).

Healthy cartilage samples had a soft and regular surface, with a constant staining of the colorant in TB and SOFG stained sections in all layers of articular cartilages. No irregularities of the surface as fibrillations or fissures were seen. The cell content was not decreased or locally increased noted as clusters (Figures 10 and 11).

In samples with signs of early OA, irregularities of the surface called fibrillation were visible. In further stages, loss of connections of the surface - so called fissures - were seen. In this stage of disease stain with TB or SOFG was absent in the upper 1/3 of hyaline cartilage (Figures 12 and 13). As OA progressed, stain loss extended into deeper layers because of depletions of PG (Figures 14 and 15). Further, cell density was decreased focal to multifocal and focal cell replications called “clusters” were seen. In these stages cracks of the calcified cartilage and pits in the subchondral bone plate were present in some of the section.

In cartilage samples graded as severe OA, stain was absent in all layers of articular cartilage (Figures 16 and 17). Further thickness of articular cartilage decreased and in some regions was reduced till the level of the subchondral bone plate, called “full-thickness erosion” (see Figure 9). A multifocal confluent to diffuse decreased cell density was visible. Also number of clusters increased in slices scored as severe OA. Further the number of cracks in the calcified cartilage and pits of the subchondral bone plate increased in cartilages with severe signs of OA (see Figure 9).

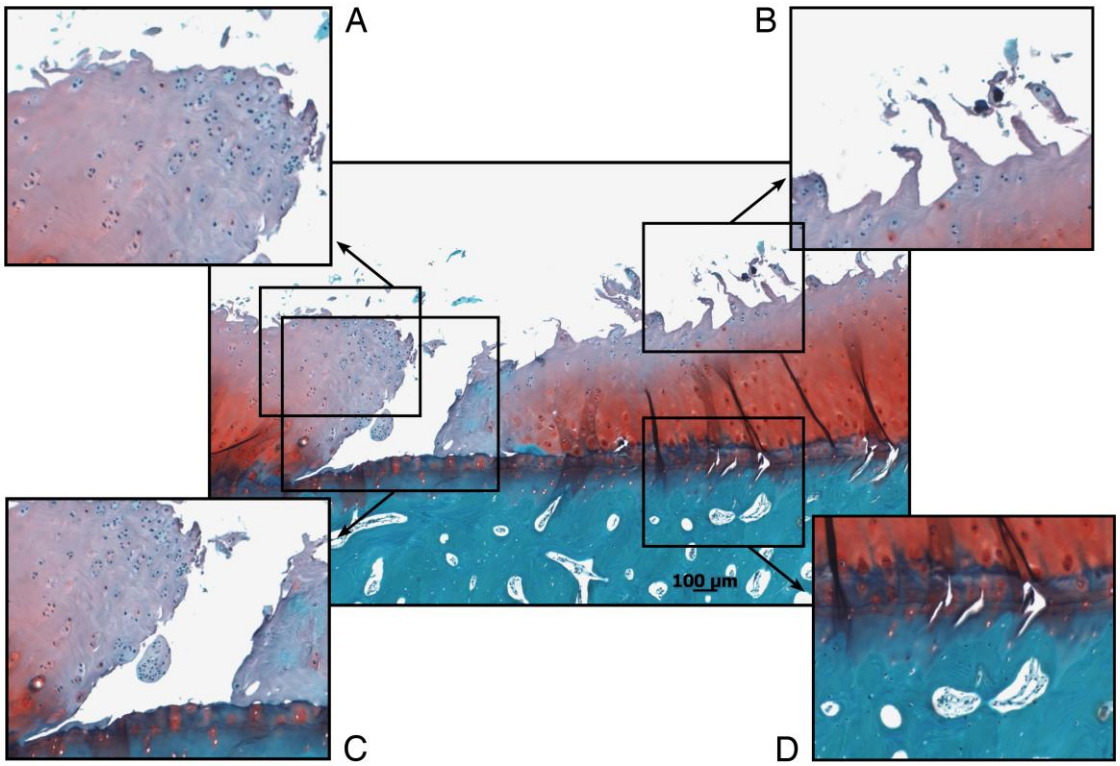


Figure 9: An example of cartilage with severe signs of OA in Safranin-O- Fast Green coloration

Chondrocytes proliferated in order to repair the destroyed cartilage, thus are seen as cluster formation ***(A).*** *Further the surface seems frayed because of the fibrillation* ***(B)****. Thickness was reduced to the level of the subchondral bone plate thus called "full-thickness erosion" (****C****). Further the calcified cartilage shows also variation, so called "cracks"****(D)****.*
Stainloss is visible because of reduced GAG content (Ranges from stainloss of the upper 1/3 (right side of the section) to stainloss of all layers of the articular cartilage (adjacent to the full thickness erosion)

4.2.2 Glycosaminoglycan content in Safranin - O - Fast Green and toluidine blue stained sections

GAG content was a part of the histologic examination by the three blinded observers as part of the modified Mankin score from Lacourt et al. Examples of the amount of staining of healthy cartilage samples, and of various grades of OA in SOFG and TB stained section are shown in the following figures (Figure 10-17).

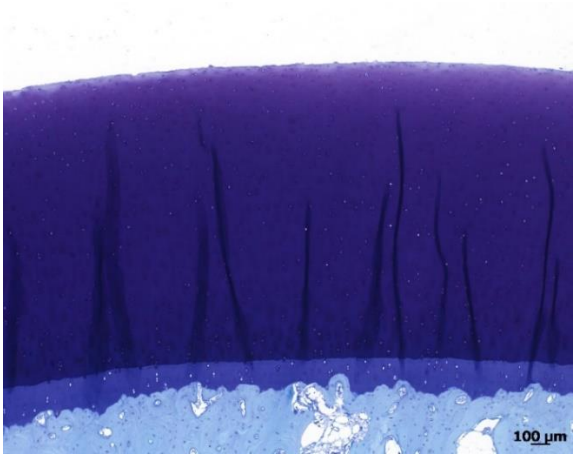


Figure 10: An example of a normal cartilage (TB)
Uniform and intense staining of all layers of hyaline cartilage.

Figure 11: An example of a normal cartilage (SOFG)
Uniform and intense staining of all layers of hyaline cartilage.

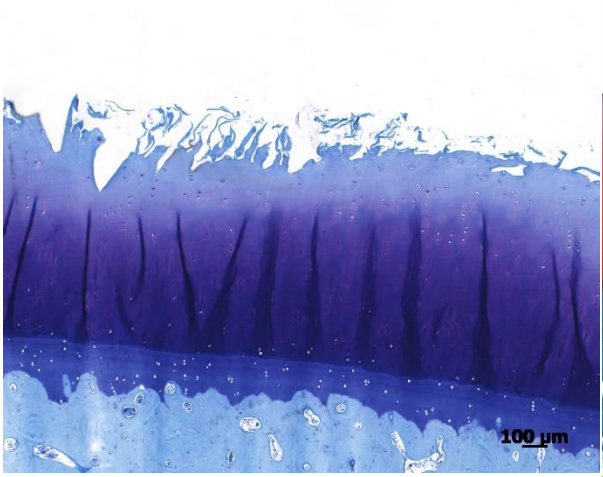


Figure 12: A cartilage sample with mild OA (TB)
Stain loss in the upper 1/3 of hyaline cartilage. Further fibrillation of the superficial cartilage layer and a fissure (see Figure 13) are visible.

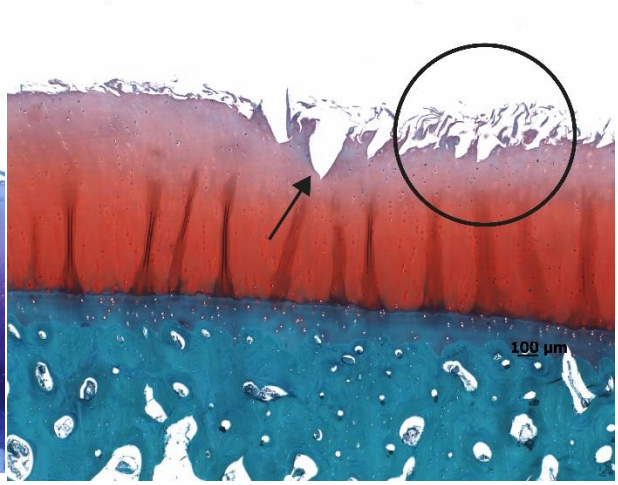


Figure 13: A Cartilage sample with mild OA (SOFG)
Stain loss in the upper 1/3 of hyaline cartilage. Further fibrillation of the superficial cartilage layer (circle) and a fissure (→) are visible.

Figure 14: A cartilage sample with moderate OA (TB)
Stain loss in 2/3 of artiuclar cartilage and fibrillation of articular cartilage surface.

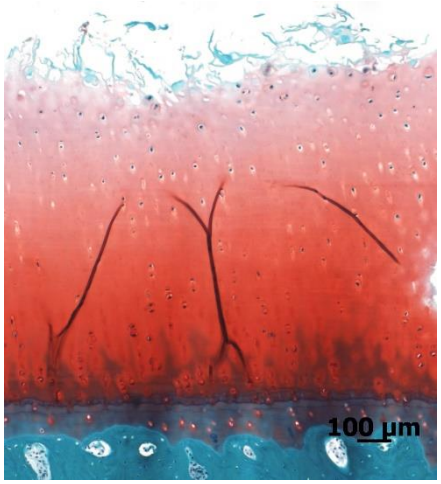


Figure 15: A cartilage sample with moderate OA (SOFG)
Stainloss in 2/3 of artiuclar cartilage and fibrillation of articular cartilage surface.

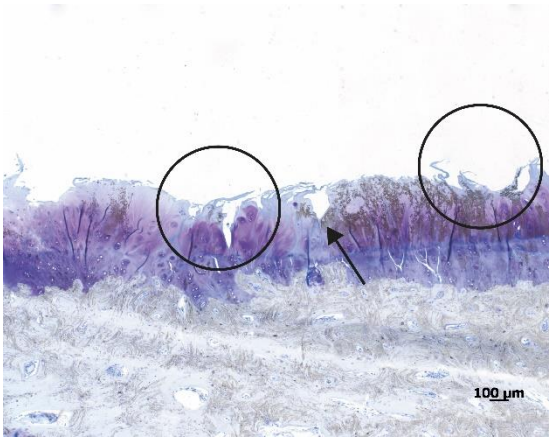


Figure 16: A cartilage sample with severe OA (TB).
Blue stain is absent in all layers of hyaline cartilage. Fibrillation (circles) of the surface, and a fissure (→) are also present.

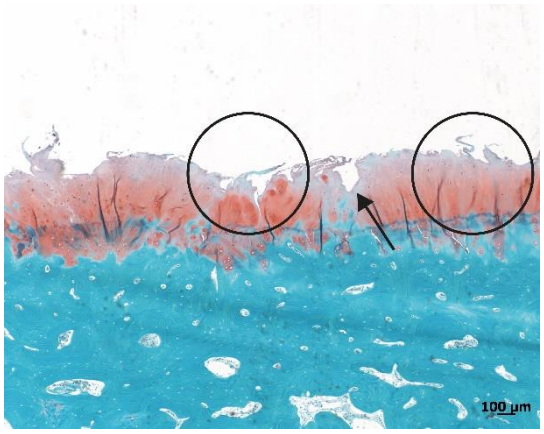


Figure 17: A cartilage sample with severe OA (SOFG).
Stain is absent in all layers of hyaline cartilage. Fibrillation (circles) of the surface, and a fissure (→) are also present.

4.3 Collagen type II content

4.3.1 Collagen type II evaluation on Picrosirius red stained sections

The ICC of the mean Picrosirius red scores between the two blinded observers was 0.81. Healthy cartilage had a smooth and regular surface with no irregularities of the surface for example fibrillation or fissures. The dense packed collagen type II fibers located on the surface of a healty cartilage sample appeared in a long waved orange color. The staining of the cartilage layers in samples with no signs of OA showed more or less homogenous staining of the section (left side, right side, middle area). Differences were observed between the different layers of the cartilage because of different package and diameter of the collagen type II fibers (Figure 18) .

Loss of integrity of collagen type II fibers resulting in absence of birefrigence lead to a black appearance. Sections showed normal birefrigence at some regions and absence of birefrigence at other regions in the same section. In samples with OA (Figure 19), the surface of the lesions showed absence of the orange birefringent zone and appeared black.

In samples with higher grades of OA the staining with the dye was inhomogenous. In regions with fissures and erosion absence of birefrigence was visible as a black area. In sections with OA the surface appeared in a yellow to greenish or black color instead of an orange color. Further signs of OA like fibrillation, fissures and erosions were visible (Figure 20, 21).

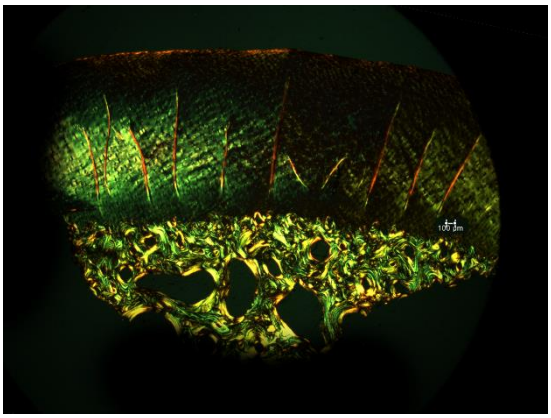


Figure 18: An example of a healthy articular cartilage sample
Dense packed collagen type II fibers of the articular cartilage surface polarized in an orange color. Further no signs of OA like fibrillation or fissures are visible.

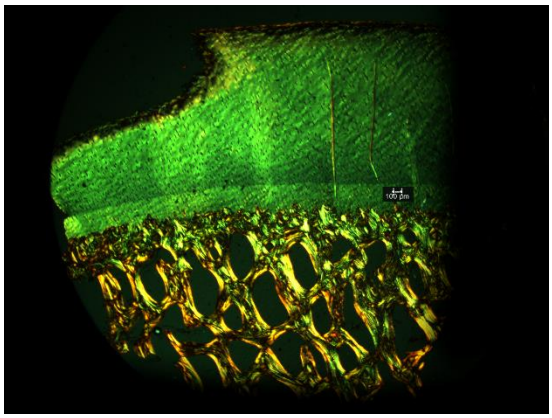


Figure 19: Cartilage sample with an erosion of 2/3 of articular cartilage surface
The surface of the lesion shows no birefringence and appears black. On the right side the surface appears orange.

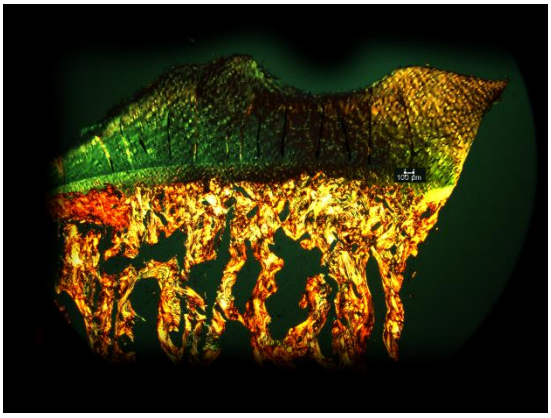


Figure 20: Cartilage sample with an erosion of the upper 1/3 of articular cartilage
The surface of the erosion shows shown loss of birefringence and appears black.

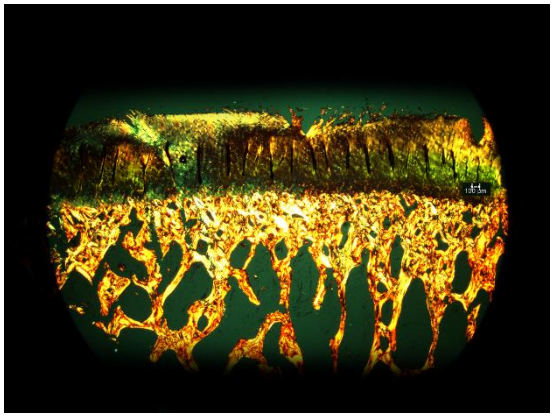


Figure 21: Sample with fibrillation of the superficial zone and a fissure
The surface shows a black color because of the loss of birefringence. Other regions show a yellow to green stain.

4.3.2 Immunohistochemical analysis of denaturated type II collagen (=collagen type II cleavage products)

The ICC of the mean immunohistochemical scores between the two blinded observers was 0.97. In healthy cartilage samples, no brownish stain was visible. Only a very light brown stain at the upper surface occurred (Figure 22). Further the surface of healthy cartilage samples appeared smooth and regular and no abnormalities of the calcified cartilage or subchondral bone plate were visible.

With increasing grade of OA, the brown stain extended in deeper zones of the hyaline cartilage (Figure 23). In such samples further signs of OA like fibrillations of the surface and cracks in the calcified bone plate were visible (see Figure 23). Further the stain degree became darker with higher grade of OA. Stain degree of Figure 24 is darker compared to the stain degree of Figure 23, while the extent of the stain is 1/3 in both samples.

In cases with severe OA, brown multifocal confluent stain was visible in all layers of articular cartilage (Figure 27). Moreover, further histological signs of OA were visible such as fissures and fibrillations and erosions (Figure 25, 26). Thickness of articular cartilage was decreased compared to the other samples, as a consequence of the progressed OA (Figure 27).

Stain degree increases with grade of OA. The brown stain extended into the deeper zones of articular cartilage in samples with higher grades of OA.

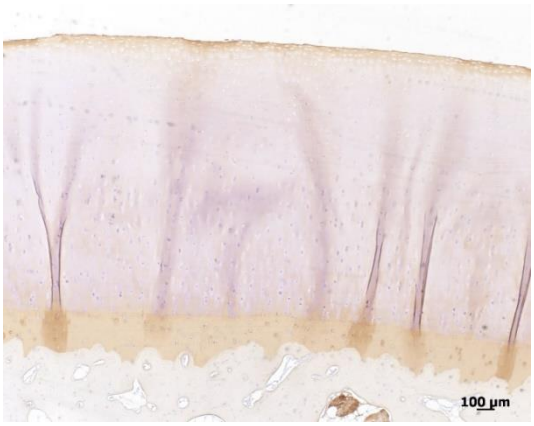


Figure 22: An example of a healthy cartilage

The surface seems smooth and regular with a light brown stain only on the upper surface.

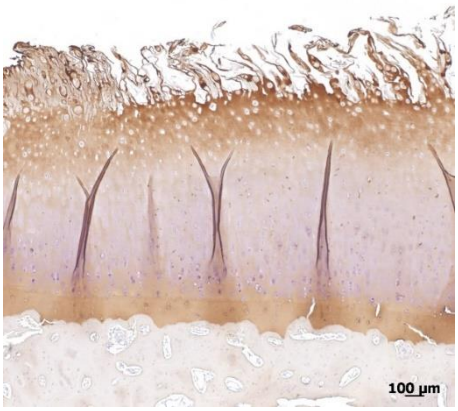


Figure 23: Articular cartilage with intense fibrillation of the surface
The brown stain is multifocal confluent in the upper 1/3 of the hyaline cartilage, with an intermediate stain degree.

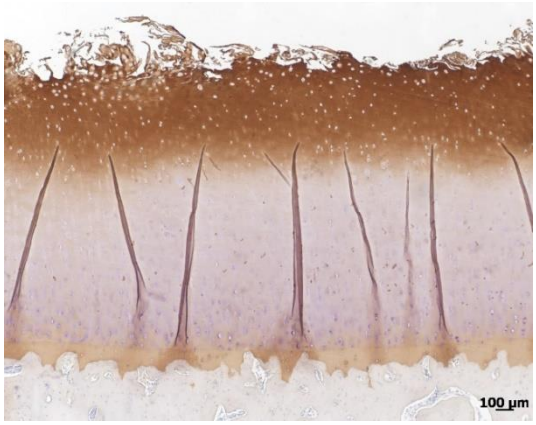


Figure 24: Sample with fibrillation of the surface of articular cartilage
Staining is multifocal confluent in the upper 1/3 of hyaline cartilage with a dark stain degree.

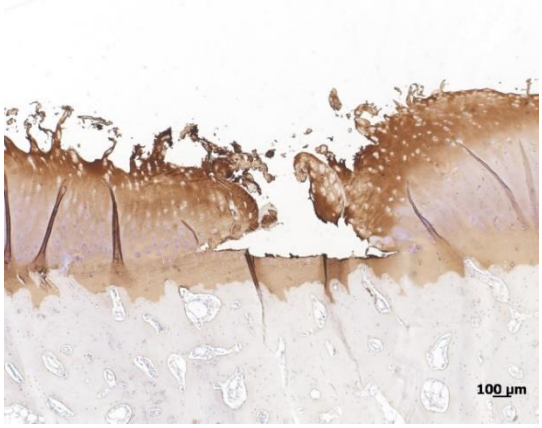


Figure 25: Full thickness erosion and fibrillation of articular cartilage
The stain uptake is multifocal confluent in the upper 2/3 of hyaline cartilage with a dark stain degree.

Figure 26: Cartilage with a deep erosion (2/3) of articular cartilage, and fibrillation of the surface
The brown staining is multifocal confluent in 2/3 of hyaline cartilage with a dark stain degree

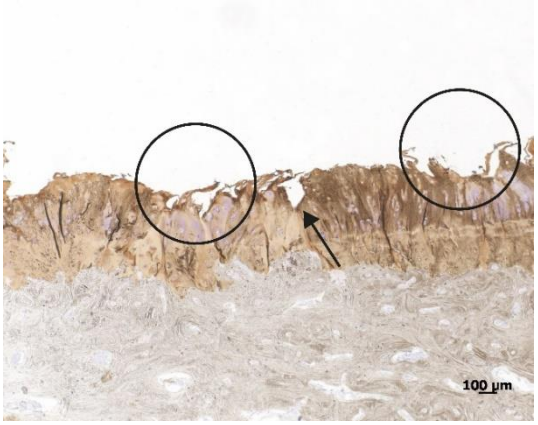


Figure 27: Cartilage sample with severe OA. *The cartilage thickness is strongly reduced, and the surface shows fibrillation. (circles). Further a fissure is noted (→). Stainuptake is diffuse in all layers of the hyaline cartilage with a dark degree of staining.*

5. Discussion

In this study the major components of the ECM: GAG, water and collagen type II were evaluated in 12 distal interphalangeal cadaver joints in horses.

The aims of the study were to describe the distribution of the major ECM components in normal cadaver distal interphalangeal joint, to identify topographical variations of the ECM components and to identify changes of the ECM components in normal compared to osteoarthritic cadaver distal interphalangeal joints.

This study showed that cartilage water content was highest and the GAG content was lowest in the middle zone compared to the palmar zones. Between the GAG and water content of the dorsal zones compared to the palmar zones no significant difference was found. In general, healthy articular cartilage (of all zones) had lower water and higher GAG content compared to samples with OA. With increasing grades of OA birefringence because of loss of package of collagen type II fibers decreased on PR stains and the amount of collagen type II product noted on immunohistochemistry increased.

5.1 GAG and water content

5.1.1 Spatial GAG and water variation of normal cartilage

To date the specific composition of the ECM of selected cartilage regions has been investigated by different researchers (Brama et al., 2000; Murray et al., 2001). However, there are no investigations on the equine distal interphalangeal joint cartilage specifically. A study of Brama et al. 2000 determined the GAG and water content of the equine fetlock joint. In the study of Brama six fetlock joints of Warmbloods were investigated. (Brama et al., 2000). Brama et al. could reveal regional differences within the normal equine fetlock joint. In the fetlock joint the highest GAG content was found in centrally located areas. The centrally located areas of the fetlock joint are under a constant load, thus leading to production of PG (Brama et al., 2000). From a clinical point the main load of the DIPJ act on central and palmar regions. The high GAG results found in the palmar regions of the distal interphalangeal joint would suggest that the palmar regions of the DIPJ are under a constant load which leads to upregulation of GAG content.

Murray et al. 2001 could also reveal regional differences of the carpal joint. In this joint highest GAG content was found in palmar regions, while lowest GAG content was found in dorsal regions. The lower content in dorsal joint regions was explained by the higher and intermittent load, which led to failure of the ECM of the articular cartilage (Murray et al., 2001). In our study GAG content of the middle zone was significantly decreased compared to GAG content of the palmar zones. Between the GAG content of the dorsal zones compared to the palmar zones no significant difference was found. This would lead to the conclusion that highest and intermittent loads occur in the central areas of de DIJP while the palmar regions are under a constant load.

In this study, GAG and water content differed significantly within the healthy distal interphalangeal joint of horses. GAG of the middle zone was significantly deeper compared to samples of the palmar zones. Between samples of the dorsal zones and palmar zones no significant difference could have been calculated. Water content behaved reversely. Thus, water content of the middle zone was significantly higher compared to the palmar zones.

ROIs from condylar samples had higher GAG and lower water content compared to samples from the intercondylar groove, which had lower GAG and higher water content. In condylar regions there is a high constant load, which leads to the production of PGs in comparison to the intercondylar region.

5.1.2 Effect of osteoarthritis on cartilage proteoglycan and water content

Water content increased with the grade of OA in our cartilage specimens. Thus, water content in this study was significantly deeper in healthy cartilage samples compared to samples with severe OA. Further water content was lower in samples with mild to moderate OA, compared to samples with severe OA. In healthy joints, the framework built by collagen type II fibers limit the amount of water, which the negative charged GAG's can attract. In articular cartilage with early OA, a disorganization and in later stages a breakdown of the collagen network occurs. This breakdown of the meshwork allows the GAG's to attract more water and thus water content increases with severity of OA (Arokoski et al., 2000; Kidd et al., 2001).

GAG content of healthy cartilage samples was significantly higher compared to samples of severe OA. Mean GAG content of cartilage with mild to moderate OA was also significantly higher compared to samples with severe OA. As mentioned in the introduction inflammation has a central role in the pathogenesis of OA. The various cytokines released because of the inflammation prevent the synthesis of the ECM, and upregulate the production of MMP's, which are able so degrade PG as well as the other components of the ECM (Frisbie 2012; Buckwalter and Mankin, 1997; Kidd et al., 2001).

5.1.3 Dimethymethylen blue assay - determination of glycosaminoglycans

Determination of GAG was done with the improved DMMB assay developed by Farndale et al. (Farndale et al., 1986). The improved assay is more specific for sulphated GAGs and has shown less interference with other polyanions. Prior to measurement, samples should be digested with papain to minimalize interference (positive or negative) with other polyanions. Papain cleaves the core protein, thus solubilizes GAGs completely. According to Farndale et al., the highest absorbance peak was found at a wavelength of 525 nm (Farndale et al., 1986). In our study absorbance was read at 525 nm with a PHERAstar©- microplate reader (PerkinElmer, Basel, Switzerland) and the analyzis of GAG was performed by Labtech GmbH in Reutlingen, Germany . To evaluate the wavelength with the highest absorbance, the maximal peak was determined on a spectrogram. In our laboratory, the highest positive peak was measured at 520 nm. Because of our findings and the suggested wavelength by Farndale et al., measurement was done at 520 nm. At this wavelength also the smallest amount of interference by other polyanions occurs (Farndale et al., 1986). Some authors describe measurement at a wavelength of 650 nm (Appleyard et al., 2003). Farndale et al. described the largest (negative) peak at 590 nm. Nevertheless, measurement at 590 nm should be avoided because of the stronger interference with other polyanions (positive or negative) (Farndale et al., 1986).

The dye, which was used in the DMMB assay has shown less stability. The complex built of sulphated GAGs and the DMMB solution will form aggregates and precipitation. Mixing or shaking will accelerate this process. It is important to measure directly after adding the dye and avoid shaking as much as possible (Farndale et al., 1986).

Some authors have determined GAG content on wet weight (Hoemann et al., 2002), and some authors on dry weight (Brama et al., 2000; Murray et al., 2001). It is not entirely clarified which method should be preferred. Lyophylisation has shown to degrade labile proteins, therefore analyses should be normalized on wet weight. A normal 1 mg sample of articular cartilage contains about 50 µg GAG and 65 – 85 % water (Hoemann 2004).

To date there are no reference values or published values of GAG and water content of the distal interphalangeal joint. This study is the first to publish such values. In our study, normal cartilage samples had a mean GAG content of 60 µg/mg calculated on wet weight and a mean water content of 71% water. Brama et al. calculated a GAG content of 43.8 ± 8.1 and water content of 67.2% ± 4.5 in the left fetlock joint, and a GAG content of 48.4 ± 8.0 and water content of 69.4 ± 2.8 in the right fetlock joint (Brama et al., 2000). The GAG and water content differs not only within a joint, but also in the different layers of the articular cartilage. The largest amount

of GAGs is found in the deep zone and superficial layers contain a lower amount (McIlwraith 2011). In this study care was taken to obtain complete full thickness cartilage samples excluding the subchondral bone. If samples are taken only from the surface, a lower GAG content is measured (Venn and Maroudas, 1977).

5.2 Collagen type II content

5.2.1 Picrosirius red

Staining of slices with PR is a method, which allows a qualitative examination of collagen fibers. Collagen type II fibers stained with PR behave birefringent, thus allowing evaluation of orientation of the fibers in articular cartilage (Vinardell et al., 2009). In this method the dye sirius red F3BA is dissolved in picric acid and is able to stain collagen fibers (Rich and Whittaker, 2005). Although quantification of collagen fibers with PR has been published, (Junqueira et al., 1979) it is more of a qualitative method (Rich and Whittaker, 2005).

In our study collagen type II fibers were assessed qualitatively with a polarizing microscope. Polarization color depends on the thickness of fibers. Dayan et al. 1989 have shown that thin fibers appear yellow to green, while thicker fibers polarize in longer wavelength thus showing an orange to red appearance. Dayan et al. however found that thickness of the fibers was not the only parameter influencing polarization color of collagen fibers. Collagen organization and package plays an important role too. Dense packed and well aligned collagen fibers also polarize in a longer wavelength (orange to red) (Dayan et al., 1989; Vinardell et al., 2009). Thus, collagen type II fibers of the superficial zones are highly ordered and are expected to polarize in an orange color. In our picture of a healthy cartilage sample fibers in the superficial zones polarized in an orange color. This fits with the expected results of highly ordered fibers of the superficial zones. Further this sample showed no signs of OA as fibrillation of the articular surface or fissures. This supports the theory that thickness is not the only parameter which has influence on the polarization of fibers. If thickness was the only parameter which had influence on the polarization the surface would be expected to polarize yellow to green.
Further samples with signs of OA had shown a yellow to green stain of the surface instead of the orange stain seen in healthy cartilage samples. This fits with the theory that loss of package of the fibers would lead to a different polarization color. In areas with integrity loss the fibers have a dark appearance because non-ordered collagen type II fibers do not behave birefringent (Dayan et al., 1989; Vinardell et al., 2009).

Further Vinardell et al. have shown that stain of the dye is homogenous in healthy cartilage samples in a horizontal direction and become inhomogenous in samples with OA (Vinardell et al., 2009). This fits with the results found in our study. Healthy cartilage samples showed a relative homogenous staining while staining became more irregular in samples with higher grades of OA. Further we observed that the samples with OA had only loss of birefringent in the areas of the lesion, while other zones of the same sample which had a regular surface polarized in an orange color and thus had shown highly packed collagen fibers. This fits with the results found by Vinardell et al. (Vinardell et al., 2009).

An advantage of this method is, that also the structure of cartilage can be examined. Beside the polarization of the collagen fibers, fibrillation, fissures and erosions are also visible, providing additional information about cartilage health.

A disadvantage of this method is, that the sample gets only a total score. A sample with loss of birefringence because of a total erosion of the whole surface, and a sample with a local 3/3 thickness erosion and a normal birefringence at the other surface gets the same score.

5.2.2 Immunohistochemistry

In this study, degradation of collagen type II content was quantified by estimating the cleavage products in immunohistochemistry with a monoclonal rabbit antibody as the primary antibody. Dodge et al. have shown that a polyclonal rabbit antibody against cyanogen bromid reacts specifically with denatured collagen type II chains or fragments of the collagen type II chains (Dodge et al.1989). This specific antibody, however does not react with intact collagen type II fibers (Hollander et al., 1994; Dodge et al. 1989). In patients with early stages of OA, degradation of collagen type II starts first in the superficial zones (Arokoski et al., 2000), thus in the early stages of OA, only superficial zones will appear brown. The protein sequence of this epitope is highly specific for collagen type II and does not cross react with other types of collagens or other proteins (Hollander et al., 1994).

In our study the surface of a healthy articular cartilage always had a light brown staining. This could be the consequence of the normal wear and tear in the articular cartilage metabolism. However, this could also be an artefact of the stain process (personal communication Prof. S. Laverty). Because the cartilage surface was smooth and constant and showed no signs of early stages of OA like fibrillation or fissures these sections were considered normal despite the light brown staining at the surface.

The stain extended in deeper zones of the hyaline cartilage with higher grade of OA. Further stain degree became darker with higher grade of OA. In cases with higher grade of OA, stain became multifocal to multifocal confluent.

In this study, the amount of staining increased with a higher content of cleavage products and this increased with the increasing severity of OA in the cartilage specimens. This presumption was confirmed by the fact that the immunohistochemistry score significantly correlated with the mean overall Mankin score.

Mean GAG content correlated negatively with the overall immunohistochemical score.

Between the mean water content and the immunohistochemical score no significant correlation was observed.

An advantage of using immunohistochemistry to describe collagen type II is that other signs of articular cartilage pathologies like fibrillation, fissures, and erosions are visible. A disadvantage of this method is, that there is no exact quantification of the collagen type II content, and we have no information about the number of crosslinks between the collagen type II fibers.

5.3. Light microscopy

5.3.1 Mankin score - histological assessment

The histological assessment in this study was done using a modified Mankin score, which has previously been used for equine cartilage by other other authors and has been proven to be consistent and reliable (Lacourt et al., 2012; Vinardell et al., 2009).

The Mankin score was developed in 1971 by Mankin et al. for the histological evaluation of articular cartilage in human hips, in patient with hip replacement due to OA or fracture of the femur head. As described in materials and methods, the original score ranges from 0 to 14 (Mankin et al. 1971). Since then this scoring system has been modified by various researchers (Pritzker et al., 2006; Rutgers et al., 2010). According to Rutgers *et al.* the histological histochemical grading system (HHGS) is a good scoring system because of the wide range of parameters, which are assessed. Further it is a widely spread used scoring system, leading to the fact that the results from different studies are comparable. A disadvantage of the HHGS is, that this system was invented for patients with severe OA, thus it works better for specimens with severe OA, than specimens with mild to moderate OA (Pritzker et al., 2006). A further disadvantage is, that there is no staging. For example a focal full-thickness erosion has the same score as a full-thickness erosion extending over the whole surface of the articular cartilage (Pritzker et al., 2006).

The scoring system, which was used in this study differed to the original HHGS in some points. Similar to the original Mankin score, four categories were analyzed. The categories were: cartilage structure, cell density, cluster formation and staining with SOFG. As a control for the amount of staining with SOFG, a healthy cartilage sample should be stained, to avoid under- or overestimation in GAG content (Pritzker et al., 2006). As done in our laboratory, staining procedure of cartilage samples should be done identically each time. Further a healthy equine articular cartilage should be used as a standard to avoid under- or overestimation in GAG content (McIlwraith et al., 2010; Pritzker et al., 2006). Unlike to the original Mankin score cluster formation was graded as a separate category. Further the integrity of the tidemark and breakdown of blood vessels were not separately recorded in the modified scoring system applied in this study.

A relative new scoring system is the OARSI system. An advantage of this system is, that it is easier to use for people with little experience in cartilage grading and this system has shown a high reliability. In contrast it is not frequently used and it is therefore difficult to compare study results to other histological cartilage studies (Rutgers et al., 2010). The OARSI system differentiates between scores and stages. The scoring is comparable to the Mankin score. It scores the extent in a vertical direction and evaluates the extent of the lesion into the different layers in the area with the highest grade of OA. The staging analyses the extent in a vertical direction and thus enables better subdivision of stages of the disease. The combination of staging and grading then gives a total cartilage score. This system was not used in our study because it has previously not been used often in horses thus results are not comparable with other studies. Because of the possibility to subdivide the stages there will be difficulties to compare the results with other studies (Pritzker et al., 2006). The modified Mankin scoring system as published by Lacourt et al. has been used often and successfully (Lacourt et al., 2012).

5.3.2 Determination of proteoglycan content

SOFG and TB are examples of colorants to stain the PGs of the articular cartilage. Depending on the stain uptake of sections PG content and regions of PG loss can be visualized. In SOFG stained sections PGs appear red to pink, while the subchondral bone is stained green. Sulfated PGs in TB blue stained sections appear purple, and collagen fibers are stained blue (Hoemann et al., 2011). In this study, the amount of stain loss in Safranin - O - Fast Green sections was assessed as a part of the modified Mankin criteria. In samples with OA, GAG content decreases (Arokoski et al., 2000) and a stain loss is visible. The degree of stain loss assessed histologically correlated with the GAG content assessed by the DMMB method. This highlights the fact that a true assessment of the PG loss may be achieved with SOFG staining only, and it may not always be necessary to determine the actual GAG concentration. Additionally using SOFG to assess stain loss we can localize the region with stain loss in the different cartilage layers and do not have a bulk measurement. When processing the SOFG section care should be taken to remove sections from the ethanol bath on time, because an elongated ethanol bath may lead to wash out of the Safranin - O - Fast Green stain and result in a false stain loss (Hoemann et al., 2011).

5.4 Limitations

One limitation of this study is that horses differed in age and sex. All specimens were collected from forelimbs, but specimens were taken from left and right limbs. Brama et al. suggests differences between left and right legs similar as in humans (Brama et al., 2000). This could not be evaluated in this study. The authors assume that by choosing Warmbloods and not Thoroughbred race horses, a similar load distribution of the right and left forelimbs would be expected. An improvement of the study design would be to collect samples from more ROIs within the joint to assess the regional differences in the biochemical parameters in more detail. A follow up study obtaining more samples spread over the complete cartilage surface would be necessary to create a complete biochemical map of the distal interphalangeal joint.

A further limitation of this study is, that the collagen type II concentration was not quantified exactly for example with an ELISA. To create a complete biochemical map of the distal interphalangeal joint the collagen type II concentration should be analysed using an ELISA as well as the hydroxyprolin crosslinks could be quantified. The advantage of our method was, that we could observe the whole cartilage and see further signs of OA, like fibrillation, fissures or erosions. Further no commercial ELISA testing systems for equine articular cartilage are available.

5.5 Conclusion

This study revealed significant regional differences of GAG and water content within the normal equine cadaver distal interphalangeal joints whereby the GAG content was higher in palmar regions than in the middle zone. Water was higher in the middle zone compared to the palmar zones. Cartilage located on the condyle was higher in GAG and lower in water content compared to cartilage located in the condylar groove. The spatial variations of GAG and water content in the joint likely reflect the load bearing characteristics of the distal interphalangeal joint. Clinically we assume that the load is higher in the palmarocentral regions compared to the dorsal regions. The palmar regions are expected to be under a constant load which lead to upregulation of GAG, while the central regions are suspected to be under a higher and intermittent load which leads to loss of GAG.

As expected GAG content decreased and water content increased when the severity of OA increased. Both parameters correlated well with the histological and immunohistochemical findings in normal cartilage and in cartilage with evidence of OA. The scoring systems were reliable and accurate for scoring OA in the distal interphalangeal joint of horses with differing degrees of OA.

6. References

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Curriculum Vitae

Vorname Name	Andrea Bachmann
Geburtsdatum	19.06.1989
Geburtsort	Luzern
Nationalität	Schweiz
Heimatort	Luzern, Aristau (AG), Menzingen (ZG)
Seit Oktober 2016	**Assistenztierärztin**, Marigin Zentrum für Tiermedizin, Feusisberg (SZ), Schweiz
April/2015- Februar/2018	**Dissertation** unter Leitung von Prof. Dr. Anton Fürst, Direktor Klinik für Pferdechirurgie, zum Thema: „Histological and biochemical characteristics of the articular cartilage of normal and osteoarthritic equine distal interphalangeal joints“ am Departement für Pferde der Vetsuisse – Fakultät Universität Zürich, Schweiz
30.12.2014	**Staatsexamen** vet. med. (Vetsuisse-Fakultät, Universität Zürich, Schweiz)
August 2014	**Masterarbei**t zum Thema: „Dermoidzysten bei einem Freiberger-Wallach“
	An der **Klinik für Pferdechirurgie**, Departement für Pferde der Vetsuisse-Fakultät, Universität Zürich , Schweiz
September/2008 – Dezember/2014	**Studium Veterinärmedizin** (Vetsuisse-Fakultät, Universität Zürich, Schweiz)
August/2002 - Juni/2008	**Matura,** Kantonsschule Alpenquai, Luzern
August/1996 - August/2002	**Primarschule** Schulhaus Wartegg/Tribschen, Luzern

List of abbreviations

ADAMTS-4= A disintegrin and metalloproteinase with thrombospondin motifs

ECM= Extracellular matrix

CI= Confidence intervals

DDFT= Deep digital flexor tendon

DIPJ = Distal interphalangeal joint

DMMB= Dimethylmethylenblue

GAG = Glycosaminoglycan

HE= Haemotoxylin and Eosin

HHGS= Histological and histochemical grading system

ICC= Intra-class correlation coefficient

IGF= Insulin dependent growth factor

IL1- ß= Interleukin 1 beta

MMP= Metalloproteinase

NB= Navicular bone

OA= Osteoarthritis

OARSI= Osteoarthritis research society international

OATS= Osteochondral Autograft Transfer System

PBS= Phosphate buffered saline

P2= Middle phalanx

P3= Distal phalanx

PG= Proteoglycans

PR= Picrosirius Red

rhIl-1= Recombinant human interleukin 1

ROI= Region of Interest

SOFG= Safranin- O- Fast Green

TB= Toluidine blue

TIMP= Tissue inhibitor of metalloproteinases

TNF-α= Tumor necrosis factor alpha

TGF-ß= Transforming factor beta

Supplements

Immunohistochemistry collagen type II protocol

Dewaxing

Xylene	2 X 5 min
Alcohol 100%	3 min
Alcohol 90%	3 min
Alcohol 70%	3 min
H_2O Milli-Q	5 min
PBS 1X	5 min

1% **Hyaluronidase (bovine testes)** in PBS

- (400µl of 10% stock solution in 4 ml 1X PBS) for 30 minutes at 37C (climate-controlled box; humid)
- Wash in 1X PBS for 2 X 5 minutes

Quencher endogenous peroxidase 3% H2O2 in methanol (60 ml jar)

- 6 ml H_20_2 30%
- 55 ml methanol
- Incubate 5 minutes (room temperature)
- Wash in 1X PBS for 2 X 5 minutes

Block non-specific sites in 20% goat serum in 30 min at RT(climate-controlled box; humid)

- 4000 ul PBS-BSA + 800 ul Goat serum

Blot the slide

Primary Antibody 1 hr incubation at 37°C (climate-controlled box; humid)

- *1/100 Collagen II* (40 uL dans 4000 uL PBS-BSA)
- collagen type II rabbit primary antibody (ab34712 Abcam inc, Cambridge, MA, USA)
- Wash in 1X PBS for 2 X 5 minutes
- Secondary antibody
- Goat anti-rabbit biotinylé Dilution 1/100
- 4 ml de PBS-BSA1%
- 20 µl goat anti-rabbit biotinyl

Incubate 45 min at room temperature (climate-controlled; humid)

Prepare the ABC complex immediately to leave at room temperature for 45 min

Wash in 1X PBS for 2 X 5 minutes

Put the ABC complex on the slides (PBS)
Leave the complex at least 45 minutes prior to applying to the tissues

Preparation of ABC complex (Vectastain kit)
Per 5 ml of 1X PBS
Add 2 drops of Reagent A
Mix well
Add 2 drops of reagent B
Mix well

(Incubate 45 minutes at room temperature) (climate-controlled box; humid)

Wash in 1X PBS for 2 X 5 minutes

DAB

5 ml Milli-Q H2O
Add 2 drops of buffer stock solution
Vortex
Add 4 drops of DAB stock solution
Vortex
Add 2 drops of hydrogen peroxide stock solution
Vortex

Put on the sections and incubate 4 to 8 minutes at room temperature

Remove the majority of DAB with the Milli-Q H2O (transfer pipette) and pour into a small disposable plastic container

Place slides in Milli-Q H2O for 5 minutes

Counterstain

hematoxylin Protocol 2 min

Rinse with water

Dip slides in acid alcohol once

Rinse with water

Put in lithium carbonate for 20 sec

Rinse with water

18. Mounting with Vectamount (aqua mount)

Products

- Xylenes, histological grade *(Sigma 534056-4L)*
- Hyaluronidase Type 1-S: from bovine testes *(Sigma H3506 lot 032K7052)*
- Methanol CH_3OH *(JT Baker (Fisher) 9093-03)*
- Hydrogen peroxide ACS 30% *(Fisher H325-500 lot 101242)*

Albumin from bovine serum, minimum 98% *(Sigma A7906)*

Peroxidase substrate kit *(Vector laboratories SK-4100) DAB*

Vectastain Elite Rabbit IgG *(Vector laboratories PK-6101)*

Hematoxylin protocol (fisher)

biotinylated goat anti-rabbit secondary antibody (Thermo scientifics, Illinois, USA),

<u>PBS 10X 1 litre</u>

- 80g NaCl
- 2g KCl
- 14,4g Na_2HPO_4
- 2,4g KH_2PO_4

Adjust pH to 7.4

Adjust volume to 1 litre

<u>PBS 1X 1 litre</u>

100 mL of PBS 10X + 900 mL H_2O Milli-Q

<u>PBS-BSA 1%</u>

0.5g BSA in 50 mL PBS 1X

<u>10% HYALURONIDASE</u>

0.3g in 3 mL PBS 1X

Mix well and divide

Keep at -20^0C

<u>1% HYALURONIDASE</u>

300 µL of 10% hyaluronidase in 3 mL of PBS 1X